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## THE SURFACE-MEDIATED UNFOLDING KINETICS OF GLOBULAR PROTEINS IS DEPENDENT ON MOLECULAR WEIGHT AND TEMPERATURE

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### ABSTRACT

The adsorption and unfolding pathways of proteins on rigid surfaces are essential in numerous complex processes associated with biomedical engineering, nanotechnology, and chromatography. It is now well accepted that the kinetics of unfolding are characterized by chemical and physical interactions dependent on protein deformability and structure, as well as environmental pH, temperature, and surface chemistry. Although this fundamental process has broad implications in medicine and industry, little is known about the mechanism because of the atomic lengths and rapid time scales involved. Therefore, the unfolding kinetics of myoglobin,  $\beta$ -glucosidase, and ovalbumin were investigated by adsorbing the globular proteins to non-porous cationic polymer beads. The protein fractions were adsorbed at different residence times (0, 9, 10, 20, and 30 min) at near-physiological conditions using a gradient elution system similar to that in high-performance liquid chromatography. The elution profiles and retention times were obtained by ultraviolet/visible spectrophotometry. A decrease in recovery was observed with time for almost all proteins and was attributed to irreversible protein unfolding on the non-porous surfaces. These data, and those of previous studies, fit a positively increasing linear trend between percent unfolding after a fixed (9 min) residence time (71.8%, 31.1%, and 32.1% of myoglobin,  $\beta$ -glucosidase, and ovalbumin, respectively) and molecular weight. Of all the proteins examined so far, only myoglobin deviated from this trend with higher than predicted unfolding rates. Myoglobin also exhibited an increase in retention time over a wide temperature range (0°C and 55°C, 4.39 min and 5.74 min, respectively) whereas ovalbumin and  $\beta$ -glucosidase did not. Further studies using a larger set of proteins are required to better understand the physiological and physiochemical implications of protein unfolding kinetics. This study confirms that surface-mediated unfolding can be described by experimental techniques, thereby allowing for the better elucidation of the relationships between the structure and function of soluble proteins as well as other macromolecules.

### INTRODUCTION

In 2004, 478,000 knee and 234,000 hip replacement procedures were conducted in the United States, accounting for a total cost of \$18.2 billion [1]. Autographs, allografts, xenografts, and metal implants are commonly used such procedures. However, these procedures have biocompatibility limitations. Metal implants, typically composed of stainless steel, cobalt-chromium alloys, and titanium-based alloys, are frequently rejected by the body's host tissue once they contact biological fluids [2]. These rejection processes are initiated primarily by the adsorption and unfolding of blood

proteins [3]. As a result of being rejected, implants typically have an average lifespan of ten to fifteen years. In addition to biological implants, the surface-mediated unfolding of proteins is important in cellular processes (e.g. enzyme activity and protein translocation), food processing, paper manufacturing, biosensors, maritime construction, and diseases such as Creutzfeldt-Jakob, Alzheimer's, amyotrophic lateral sclerosis, cancer, and osteoporosis. However, little is known about the mechanisms behind this type of protein unfolding. Modeling the rates at which proteins unfold has become one of the most prominent challenges in computational biology and chemistry.

Adsorption and unfolding processes are related to the molecular configuration and dynamics of the bound macromolecule. More emphasis has recently been placed on the unfolding of soluble proteins than other bio-macromolecules because proteins are far more diverse in structure than DNA, RNA, or lipids [4, 5]. Many soluble proteins are globular and composed of internalized hydrophobic domains joined by hydrogen bonds, disulfide bridges, and hydrophobic interactions [6]. To remain soluble in aqueous media, hydrophilic functional groups enclose the hydrophobic inner core. Because of functional variability, many proteins must be dynamic and flexible, transforming into different conformations to facilitate environmental adaptation at the cost of intramolecular bonding [7, 8].

Adsorption to solid supports occurs due to favorable entropic increases in hydrophobic interactions induced by dehydration between the protein and surface [9]. To assist adsorption and environmental adaptation, proteins unfold and expose more active sites that participate in subsequent interactions [10]. In general, proteins of higher molecular weight have more surface contacts, more flexibility because of the increased total degrees of freedom, and therefore unfold faster than smaller proteins [11].

Unfolding kinetics models have been developed for proteins in solution [12–15]. The structure of globular proteins is commonly based on a rigid sphere composed of network glass due to similarities in high packing density values [16–19]. Computational models have determined the maximum distance over which interactions can occur between a solid surface and a protein functional group to be between 20 and 30 Å [20]. This distance defines a zone of influence to help us better understand the surface-mediated unfolding process. After the protein functional groups within this zone of influence have responded to attractive forces and diffused onto the interface, reversible protein denaturation (rupture of the internal bonds) occurs [21, 22]. Because of adsorption and the initial unfolding at the interface, structural stability and solubility decrease, resulting in the release of side chain groups [23]. Solvent molecules then enter the interior of the protein core, further reducing secondary structure [24, 25]. Following the passage through one or more transition states, irreversible spreading leads to proteins becoming fully denatured and relaxed [26]. In fibrinogen, spreading on hydrophobic surfaces results in an increase in footprint size from 100 nm<sup>2</sup> to 500 nm<sup>2</sup> during a single-exponential decay of 1735 s [27]. Finally, interfacial protein aggregation, multiple film formation, and/or gelation may result depending on protein structure and function [28, 29]. It is important to note that these models do not apply to surface-mediated unfolding because the physical processes differ significantly.

Although much is still to be learned about surface-mediated protein unfolding, models describing such mechanisms are important because many biochemical reactions are surface-mediated, such as cellular processes taking place at membranes. We are also aware that surfaces catalyze protein unfolding such that unfolding on surfaces occurs at a lower temperature than it would in bulk solution [30]. The rates of unfolding on most synthetic, rigid surfaces are slow because only the protein is allowed flexibility relative to the solid surface [31, 32]. It is likely that unfolding or relaxation of a protein at a membrane surface is much faster because both components

are flexible [33]. And, it is easy to imagine that at physiological temperatures, flexibility is optimal to match performance.

The separation, quantification, and partial characterization of proteins by liquid chromatography is a highly respected and common process. Hydrophobic interaction high-performance liquid chromatography (HPLC) has proven to be not only a good means by which to separate proteins, but also a method to determine their relative hydrophobic properties [34, 35]. It is now well accepted that the retention properties of cytochrome c increase with time and temperature on ionic and hydrophobic supports [36, 37]. Furthermore, loss of protein by HPLC has implications on the usefulness of this method for quantification in addition to studying surface-mediated unfolding. With the use of ion-exchange and hydrophobic interaction HPLC of fibrinogen, cytochrome c, bovine serum albumin (BSA), and immunoglobulin G (IgG), Goheen and Gibbins identified a logarithmic relationship between protein loss and molecular weight [11]. In the present study, the protein losses for the globular proteins myoglobin, ovalbumin, and  $\beta$ -glucosidase were compared with earlier findings. We investigated this by absorbing proteins to non-porous cationic polymer beads under different gradient residence time conditions and tested the null hypothesis that protein loss, and hence unfolding kinetics, is independent of molecular weight.

## MATERIALS AND METHODS

The Bio-Rad Laboratories (Hercules, CA, USA) HPLC dual piston, dual-pump gradient system was used for protein separation. The 2.5 mL mixer of this system was replaced with a low volume Lee Co. 250  $\mu$ L visco-jet micro-mixer (Westbrook, CT, USA). Ultraviolet/visible absorbance was obtained with a 9  $\mu$ L flow cell at 280 and 409 nm to detect proteins. Microsoft-based BioRad ValueChrom software recorded chromatograms and integrated peak areas.

A MA7Q (quaternary amine) anionic exchange column from Bio-Rad was used and included nonporous, spherical, polymeric beads. The column has a loading capacity range of 5–10 mg for static proteins and 1–2 mg for dynamic proteins. For residence time studies, the temperature was kept constant at 37°C. The temperature was varied for select experiments using a Bio-Rad column heater. The column was kept in an ice bath for 0°C sorption experiments.

Chemicals and reagents were of the highest purity available from Sigma (St. Louis, MO, USA) unless otherwise indicated. Water used was deionized and purified with a Milli-Q filtration system (Millipore, Bedford, MA, USA). Buffer A contained 20 mM tris (hydroxyl-methyl) aminomethane (Tris)-HCl base (Trizma, reagent grade). After filtration through a 0.2- $\mu$ m membrane (Whatman, Maidstone, UK), the pH was brought to 8.5 using 0.1 M NaOH. Buffer B contained 20 mM Trizma base and 500mM NaCl ( $\geq 99\%$  purity, ACS grade). After filtration through a 0.2- $\mu$ m membrane, the pH was brought to 8.5 using 1 M HCl.

Sample preparation for myoglobin (horse heart,  $\geq 90\%$  purity, Sigma), ovalbumin (turkey egg, Grade VI, Sigma), and  $\beta$ -glucosidase (almond, 30 units/mg, Sigma) consisted of solubilizing the protein in Buffer A (pH 8.5), unless otherwise noted. Protein solutions

were prepared daily to concentrations of 1 mg/mL. Solutions were stored at 8°C when not in use and warmed to room temperature prior to analysis.

HPLC gradients initiated with Buffer A and ended with Buffer B. The time between sample injection and the start of the gradient varied between gradients (Table 2). 100% recovery was determined using a low-dead-volume (LDV) connector (and 0.5 M NaCl) in place of a column. Gradient 0 was a control in which an isocratic gradient of 0.5 M NaCl should have prevented all protein binding to the cationic support and 100% recovery was anticipated. For all gradients, with the exception of gradient 0, NaCl concentrations increased linearly over 15 min from 0 M to .5 M (0.03 M/min). The column was washed for 5 min with Buffer B after the completion of the gradient in order to ensure elution of all recoverable proteins. The flow-rate was kept at 1 mL/min and a sample injection volume of 20 µL was used for all experiments.

For each experiment, absorbance readings at 280 nm and 409 nm were recorded using a Gilson 118 ultraviolet/visible detector (Gilson, Middleton, WI, USA). These recordings monitored protein loss and ensured the elution of all proteins from the column. Absorbance at 409 nm was used to verify the components of myoglobin. Protein peaks were integrated using the Bio-Rad ValueChrom system. The adjusted retention time was calculated as the time difference between protein injection and elution. In order to calculate and compare protein recovery, a 9 min elution time was arbitrarily chosen to measure protein levels. Consequently, protein recovery was calculated as the ratio of  $(\text{area}_{\text{min}}) / (\text{area}_{\text{dead volume}})$ .

The column was rejuvenated periodically by eluting a 0.1 M NaCl and/or 0.1 N NaOH solution for 1 hour. Afterward, Buffer A was eluted until equilibrium was reached between the mobile and stationary phases before another experiment could be conducted.

## RESULTS

### Protein Retention and Recovery

The unfolding kinetics of myoglobin,  $\beta$ -glucosidase, and ovalbumin were examined in this study. Some characteristics of these proteins are shown in Table 1. Protein-support residence times were varied in order to examine surface-mediated protein unfolding kinetics on the cationic support. Protein loss with respect to molecular weight was calculated as described in the Materials and Methods. Subsequent data will help delineate the mechanisms of protein adsorption and unfolding at the protein-surface interface.

When a 15 min (1.0 mL/min) linear gradient was started immediately after each

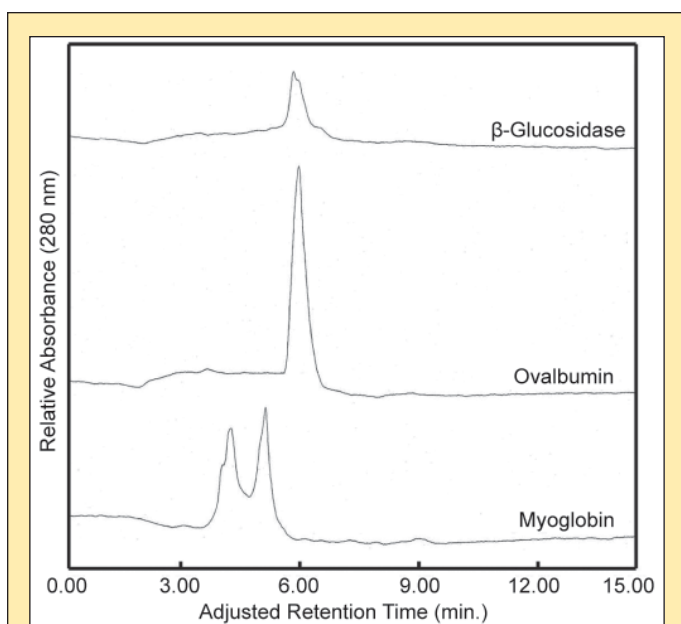
of the proteins in Table 1 was injected, profiles shown in Figure 1 resulted. Ovalbumin and  $\beta$ -glucosidase were each characterized by a single primary peak eluting from the column approximately 6.5 min after the start of the gradient. Myoglobin had two prominent peaks, with the first peak eluting either as a doublet or a singlet with a left-side shoulder component. At a wavelength of 409 nm, the first peak to elute from the column during gradient 1 (37°C) had a retention time equivalent to the second component of this doublet (approximately 4.53 min). This peak was assigned as holo-myoglobin. The first member of the doublet (or shoulder component) was assigned apo-myoglobin. The peak eluting after the doublet was assigned as a dissociated heme prosthetic group because the retention and area of this peak did not change significantly throughout our experiment, and its absorbance was strong at 409 nm.

Figure 2 shows the absorption spectrum of myoglobin, ovalbumin, and  $\beta$ -glucosidase when gradients 1, 2, 3, and 4 were used at 37°C. See Table 2 for a description of the different gradients used. For myoglobin, increasing the residence time reduced the distance separating the two major peaks and increased retention time (4.87 min and 5.49 min, 0 min and 30 min, respectively). Initial exposure to the column (gradient 1) resulted in a protein loss of 62.1% to the surface when compared to the LDV connector (gradient 0). At the longest residence time (gradient 4), the initial holo-myoglobin peak was almost absent, with an 84.7% loss.

Protein	MW (kDa)	pI	Function	% $\alpha$ -helices and $\beta$ -sheets	Internalized Disulfide bonds?
Fibrinogen	330	5.1	Blood coagulation	Chain A,D: 23% helical, 0% beta sheet; Chain B,E: 33% helical, 18% beta sheet; Chain C,F: 33% helical, 19% beta sheet.	Yes
Immunoglobulin G	150	6.1-8.5	Antibody in the blood against bacteria and viruses	3% helical, 43% beta sheet	Yes
Bovine Serum Albumin	69.9	4.9	Lipid binding	67% helical, 0% beta sheet	Yes
$\beta$ -glucosidase	60.4 <sup>a</sup>	4.5	Cellulose digestion	40% helical, 16% beta sheet	No <sup>b</sup>
Ovalbumin	42.9	4.5-4.9	Unknown	28% helical, 31% beta sheet	Yes
Myoglobin	17.1	6.9/7.35	Oxygen storage	71% helical, 0% beta sheet	No
Cytochrome C	11.8	9.1	Electron carrier involved in aerobic energy generation	20% helical, 11% beta sheet	Yes

<sup>a,b</sup>  $\beta$ -glucosidase is a homodimer consisting of two monomers of approximately 65,000 Da molecular weight. The native molecular weight of the enzyme is 130,000 Da. Although no disulfide bonds maintain its internal structure, a disulfide bond links the two dimers to form the native structure.

**Table 1.** Representative characteristics of the proteins analyzed in this study. Protein data are from modeling tools available at the ExPASy proteomics server (<http://ca.expasy.org/tools/>), unless otherwise noted.

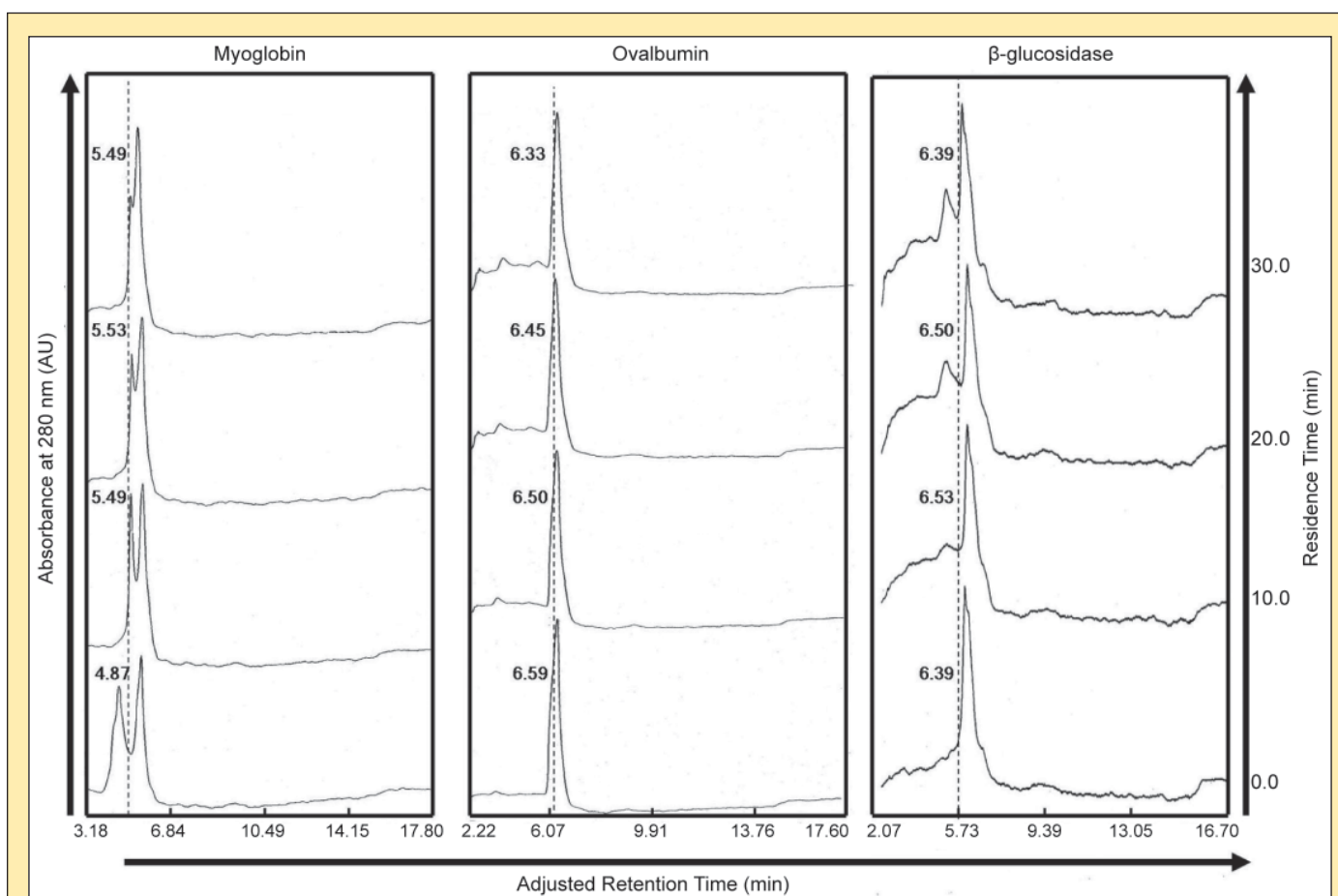


**Figure 1.** Representative chromatographic profiles of myoglobin, ovalbumin, and  $\beta$ -glucosidase adsorbed to a cationic surface using gradient 1 at 37°C.

Increasing the residence time did not increase the retention times of either ovalbumin or  $\beta$ -glucosidase. However, both exhibited a loss in protein to the surface. For ovalbumin, 33.7% of the protein was initially lost at gradient 1. After a 30 min gradient delay, protein levels were further reduced, resulting in a 43.3% total loss. Overall, ovalbumin exhibited a slight decrease in retention time with increasing residence (6.59 to 6.33 min, 0 to 30 min, respectively). Similarly,  $\beta$ -glucosidase yielded an initial reduction of 29.6% at gradient 1. For gradient 4, a total of 56.3% of protein was lost.

At 9 min, 71.8%, 31.1%, and 32.1% of myoglobin,  $\beta$ -glucosidase, and ovalbumin were lost to the column surface, respectively. The trends in protein loss with respect to residence time over the 30 min time range are illustrated by an exponential relationship in Figure 3 ( $R^2$  values for myoglobin,  $\beta$ -glucosidase, and ovalbumin are 0.997, 0.997, and 0.957, respectively).

A comparison of protein recovery was performed. The logarithm of the molecular weights of myoglobin,  $\beta$ -glucosidase, and ovalbumin, in addition to cytochrome c, BSA, IgG, and fibrinogen, were plotted against their respective percent losses to the surface at 9 min. A positively increasing linear relationship ( $R^2$  value of 0.970) was observed for most proteins, with higher molecular weights resulting in lower recovery (Figure 4). Myoglobin was the only protein which significantly differed from this trend.

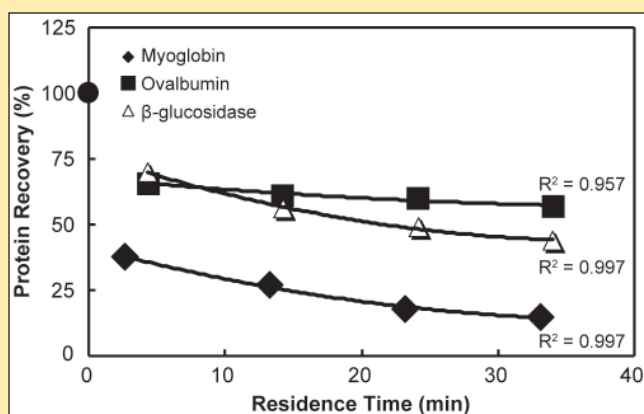


**Figure 2.** Chromatographic profiles of myoglobin, ovalbumin, and  $\beta$ -glucosidase adsorbed to a cationic support using gradients (bottom to top) 1, 2, 3, and 4 (37°C). The variable residence time,  $t_r$ , subtracted from the elution time, normalized the data relative to the start of the Buffer B (0.5 M NaCl) linear gradient. An increase in retention time is observed as the adjusted residence time is increased for myoglobin.

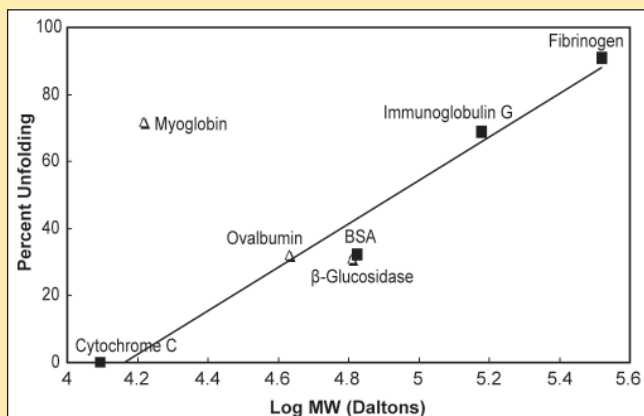
Gradient No.	$t_1$ (min)	Retention at 37°C (min)		
		Myoglobin	Ovalbumin	$\beta$ -glucosidase
0	N/A	~0	~0	~0
1	0	4.81–4.87	6.52–6.59	6.39–6.60
2	10	15.49–15.51	16.43–16.50	16.53
3	20	25.53–25.55	26.41–26.45	26.39–26.51
4	30	35.44–35.49	36.32–36.33	36.28–36.39

<sup>a</sup>Gradients used in these experiments are labeled 0–4.  $t_1$  is the delay in time between sample injection and the start in a linear gradient of Buffer B (0.5 M NaCl). All gradients were 15 min in length (1 mL/min) and for each a range of retention times were observed for the three studied proteins. Total protein amounts (100% recovery) for each sample were determined by an isocratic condition of Buffer B only (gradient 0) using a LDV connector in place of a column

**Table 2.** Variable time  $t_1$  for gradients<sup>a</sup>.



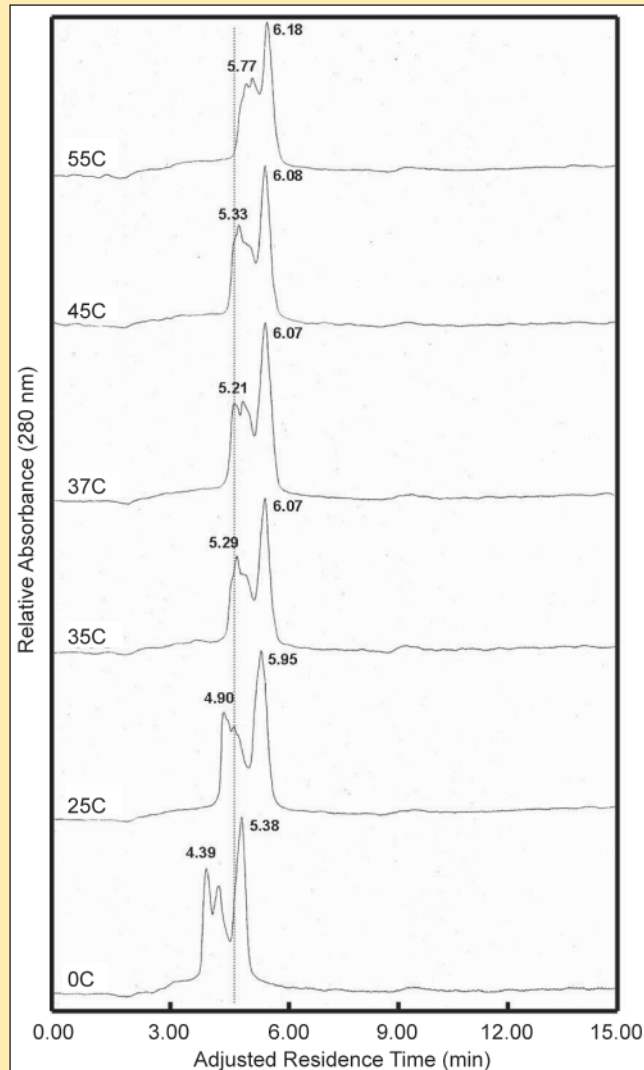
**Figure 3.** Recovery percentages of myoglobin, ovalbumin, and  $\beta$ -glucosidase as a function of variable residence time. Protein recovery, the total concentration eluted from the column, was calculated by taking the integral of obtained peaks. For myoglobin, ovalbumin, and  $\beta$ -glucosidase, a decrease in eluted protein occurred as variable residence time increased from gradient 1 to 4 at 37°C. Myoglobin exhibited high surface-mediated unfolding rates, with recovery decreasing shortly after exposure to the cationic support.



**Figure 4.** The unfolding percentage as a function of molecular weight for proteins adsorbed to cationic surfaces at 37°C. The percentage of protein unfolded was determined by the percent of protein loss. Losses were calculated at an arbitrary 9 min elution time for each protein. A linear relationship exists between the proteins, with an  $R^2$  value of 0.970.

## Myoglobin Unfolding and Temperature

The effects of temperature on surface-induced losses were investigated with myoglobin. Figure 5 shows that as the temperature increased, retention time also increased (0°C to 55°C, 4.39 min to 5.77 min, respectively), bringing the elution of myoglobin and apo-myoglobin closer to that of the heme-prosthetic group. At the highest examined temperature of 55°C, approximately 89% and 63% of apo-myoglobin and myoglobin were recovered respectively using an anion exchange column compared to the lowest temperature (0°C).



**Figure 5.** Myoglobin (horse heart) separation on a cationic surface as a function of temperature using gradient 1. Retention times of the myoglobin peak increased with elevated temperatures, indicating higher levels of surface-mediated unfolding.

## DISCUSSION

In the present work, the unfolding kinetics of  $\beta$ -glucosidase, ovalbumin, and myoglobin were studied to determine if unfolding rates were related to protein molecular weight. These proteins were chosen because of their wide range of structural and functional properties.  $\beta$ -glucosidase assists cellulose digestion by cleaving the breakdown products of endoglucanase and exocellobiohydrolases into two glucose molecules [38]. Ovalbumin is a glycoprotein abundant in avian eggs (comprising 60% of the total protein in egg whites) whose function is yet to be deciphered [39]. Myoglobin, found typically in muscle tissue, is a small monomeric protein involved in oxygen storage.

The retention time for myoglobin on a cationic support increased with temperature (Figure 5). This alteration in retention time may be the result of increased exposure to ionized regions of the protein. Khechinashvili et al. suggest that at room temperature, globular proteins are stabilized by the entropy of non-polar group hydration and enthalpy as a result of internal interactions [40]. Because of the substantial heat capacity associated with nonpolar group hydration, high temperatures lead to the decrease in its stabilizing capacity and an increase in destabilizing forces such as conformational entropy and vibrational effects. The side chains of a protein will rotate and bend more as the temperature is raised, resulting in an increase of exposed sites for attachment.

The observed increases in retention time and decreases in percent recovery for myoglobin are contrary to previous studies which illustrate higher elution temperatures result in decreased retention times [41]. These experiments were conducted under acidic conditions using silica-based supports. The experiments discussed in the present study were performed at near-physiological conditions to explore biologically significant protein behavior rather than developing optimal separations.

Other than temperature, the recovery of proteins as a function of sorbent residence time was also analyzed. A comparison of the proteins revealed a correlation between recovery and molecular weight. Increases in the logarithm of molecular weight led to lower recovery in a linear correlation for all proteins with the exception of myoglobin (Figure 4).

Previously we suggested the surface acts as a catalyst for protein unfolding. As residence time is increased before the start of each gradient, the proteins have increasing amounts of time to find the surface-aqueous interface. However, whether surface-mediated unfolding is solely related to protein flexibility and stability is still not certain. Further experiments should be carried out to more fully describe the unfolding phenomena on solid supports.

All of the proteins examined fit the linear trend between unfolding and molecular weight except myoglobin. On average, the retention time of myoglobin differs significantly from ovalbumin and  $\beta$ -glucosidase for all gradients (Table 2). Myoglobin also exhibited a high degree of unfolding, with an increase in retention time and decreased percent recovery as residence times were extended (Figures 3 and 4). This result may be related to the structure and function of myoglobin. Although myoglobin contains only 153 residues, 71% of its secondary structure is alpha helices (eight right handed alpha helices). While BSA has a similar percentage

of helices (67%), its internal structure is maintained by disulfide bridges, which are absent in myoglobin. Disulfide bridges can be vital in maintaining structural integrity [42]. Although the combination of these two characteristics may result in a relatively flexible structure for myoglobin, they may not be the only factors involved.  $\beta$ -glucosidase also does not possess internal disulfide bridges, yet its structural flexibility is consistent with the general unfolding trend. Another reason for the deviation may be due to the relatively high isoelectric point (pI) of myoglobin (6.9/7.35) compared to the other proteins (approximately 5.0). Because the buffers used were equilibrated to pH 8.5, myoglobin could have had a disproportionately stronger affinity for the cationic surface. This affinity would unlikely be the sole result of net charge since myoglobin (-1.3) is not as negatively charged as other proteins at this pH (-15.2, -35.2, and -17.1 for ovalbumin,  $\beta$ -glucosidase, and BSA, respectively), and consequentially will not be as attracted to the positively charged surface. The charges of IgG and fibrinogen could not be calculated due to the lack of structural knowledge.

Interestingly, although myoglobin and cytochrome *c* are both low molecular weight proteins, their unfolding rates differ significantly. Under our experimental conditions, cytochrome *c*, which is a soluble protein associated with the mitochondrial inner membrane, has almost no unfolding on the cationic support (Figure 4). It is possible that this is also related to the pI of the proteins because cytochrome *c* has a charge of 6.6 under our variables. Another factor involved may be the protein's hydrophobic characteristics. Myoglobin and cytochrome *c* have different relative hydrophobicity values of 0.23 and 0.0, respectively [43]. Because hydrophobic interactions are entropically favored by protein adsorption, this may lead to increased unfolding. These relationships need to be examined in greater detail in order to develop a more comprehensive model.

Recent studies have suggested numerous reasons and models for the unfolding of proteins under various conditions such as temperature, pH, and pressure [44, 45]. In a related analysis, the adsorption and unfolding of proteins at interfaces was investigated based on computational models. It was found that the process of unfolding occurs via a generic, rapid loss of native contacts, rather than as a gradual event [46]. Although other theoretical approaches have occurred, only a few studies have experimentally examined surface-mediated protein unfolding [47]. Of these, most deal with the examination of a single protein and not a comprehensive comparison between many different proteins.

In the present investigation, HPLC was used to analyze and compare a wide range of proteins with various molecular weights, structures, and functions. It has been demonstrated that there is a positively increasing linear trend between molecular weight and the rate of unfolding. This may be due either to the surface acting as a catalyst or protein destabilization as a result of sorbent binding. The present study confirms that surface-mediated unfolding can be described by experimental techniques, thereby allowing for the better elucidation of the relationships between the structure and function of soluble proteins as well as other macromolecules. In practical application, this knowledge will assist in unraveling the origins of protein-related diseases such as Creutzfeldt-Jakob, Alzheimer's, amyotrophic lateral sclerosis, and cancer.

## ACKNOWLEDGEMENTS

The authors thank the U.S. Department of Energy, Office of Science, Science Undergraduate Laboratory Internship program at the Pacific Northwest National Laboratory. We also thank James Campbell, Eric Hoppe, and Tere A. Simmons for their assistance in the laboratory and Karen Wieda, Craig Gabler, Royace Aikin, and Dale Johns for running excellent science education programs.

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